## REMARKS

Claims 30-49 are pending in the application. Claims 30, 32-35, 39, 41-43, 47, and 49 have been amended to better describe the invention and for consistency reasons. Since the amendments place the application in condition for allowance, remove issues in the event of an appeal, and/or do not require further searching, entry is respectfully requested. Favorable reconsideration in light of the amendments and the remarks which follow is respectfully requested.

### The Amendments

The independent claims have been amended to better describe the nature of the invention as having multiple constituents composed of more than one chemical species derived from the outer layer of blood or mesothelial cells. The term "the outer layer" refers to the glycocalyx of said cells as explained in the description and cells have only one glycocalyx so that there is one or "the" outer layer.

### The Enablement Rejection

Claims 30-49 have been rejected under 35 U.S.C. § 112, first paragraph, for enablement reasons with regard to native heparan sulfate and the transitional phrase. Referring to the arguments presented by the Applicants in the RCE Submission in June 2005, the Examiner asserts that the trace impurities of the native heparan sulfate are not disclosed or claimed. Applicants respectfully disagree to the extent that the impurities have been disclosed. The claims have been amended to more clearly reflect the nature of the invention.

Initially, it is noted that the RCE Submission of June 2005 states "[t]he native heparan sulfate materials contains trace impurities that come from other constituents of the outer layer of a red blood cell and/or mesothelial cell." In this sense, the claims have been amended to reflect that the invention makes use of more than one constituent derived from a mesothelial and/or blood cell. Thus, the subject "impurities" are indeed expressly covered by the amended claim language, which requires

"constituents of the outer layer of a blood cell, constituents of the outer layer of a mesothelial cell." The change of singular to plural, i.e. the change from "constituent" to "constituents" should be allowable since the claims as originally filed clearly contain the wording "constituents". The claims as originally filed are attached. However for a reason we cannot follow the plural term was changed to singular without any necessity to do this.

The term "isolation" (Examples 1-4) is a term of art in the biological sciences. As stated in the RCE Submission of June 2005, this invention is distinguished by the use of "native heparan sulfate materials." Native biomolecules are functional and have biological activity; such native biomolecules are functional due to specific and general affinity for binding to other species in the biological milieu that is due to their native tertiary structure. The traditional chromatography and precipitation techniques described in Examples 1-4 are universally understood to only ENRICH the targeted group of biomolecules relative to the other elements in the biological milieu. It is a common term of art in the biological sciences to refer to a sample that has been enriched with a particular component to a high degree (for example, at least 80%) as having "isolated" that particular constituent. It is recognized as a virtual impossibility to purify from endogenous tissue proteins, glycoproteins, DNA, RNA, etc. to 100% purity without using harsher techniques that destroy their native structure. Even recombinant proteins expressed with high binding affinity tags cannot be purified from expression hosts to 100% purity and DNA precipitation never yields DNA that is completely free of contaminating proteins and histones.

Moreover, Examples 1 and 3 expressly describe in a manner sufficient to teach one skilled in the art how to isolate such constituents. That is, the methods of preparing the mixture of glycocalyx "constituents", i.e. the mixture of the constituents of the outer layer of blood or mesothelial cells of the claims as described in the specification contains the subject impurity materials. As described by the specification, the preparation of constituents of the outer layer of a blood cell and/or constituents of the outer layer of a mesothelial cell result in a final fraction that contains MULTIPLE

constituents, some of which can include the subject "impurities". Whatever impurities are present in the materials are derived from the disclosed mesothelial and/or blood cells and are consistently present as the inevitable byproduct of the preferred embodiments in the specification. No experimentation needs to be done to prepare the materials required for the invention. One reasonably skilled in the art knows that impurities are present in any *in situ* preparation.

Concerning the term "impurities" it has to be stressed that the mixture of constituents of the outer layer of mesothelial cells or blood cells does not contain "impurities" per se. A basic idea of the invention relates to transfering the glycocalyx of a mesothelial cell or a blood cell, i.e., to transfer the complete constituents (= all constituents) of the outer layer (= glycocalyx) of a mesothelial cell or of a blood cell, which have to be hemocompatible to an artificial or natural surface in order to make this surface hemocompatible. Consequently, all constituents of the outer layer (glycocalyx) of a mesothelial cell or of a blood cell are used since all these constituents have to be hemocompatible. The invention is uses easily obtainable mesothelial cells or blood cells which are enzymatically shaved (i.e. the constituents of the outer layer of these cells are cleaved enzymatically) and thereafter the intact cells but without glycocalyx are separated from the glycocalyx constituents and after removing the enzyme solution the mixture of all glycocalyx constituents are used as coating material for surfaces of, for instance, medical implants such as stents in order to make these surfaces blood-compatible (=hemocompatible). Therefore, the claimed mixture of constituents of the outer layer of a mesothelial cell or of a blood cell does not contain "impurities"; instead, the mixture contains all single constituents of the outer layer while it could be that some of these constituents are not exactly characterized. However there may be some constituents which are not fully chemically characterized, but these constituents are part of the undoubtfully hemocompatible native outer layer and thus of the invention and should not necessarily be regarded as "impurities".

Once again, a basic aspect of the invention is to isolate all constituents of the outer layer (glycocalyx) of a mesothelial cell or of a blood cell and to transfer this

hemocompatible outer layer to a surface of an artificial or natural material in order to make the surface hemocompatible. Within this process all constituents (chemically characterized or not) are used as coating material and not only a single constituent which may comprise "impurities" in form of other minor constituents of the outer layer of a mesothelial cell or blood cell.

Finally, as noted by MPEP § 2111.03, the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. *In re Gray*, 53 F.2d 520, 11 USPQ 255 (CCPA 1931). MPEP § 2111.03 further notes *Ex parte Davis*, 80 USPQ 448, 450 (Bd. App. 1948) that clearly explains that "consisting of" functions to close the claim to the inclusion of materials other than those recited except for impurities ordinarily associated therewith.

The "constituents" of the claims are adequately described in the specification in a manner so that one skilled in the art can MAKE and USE the constituents, so long as the constituents come from the outer layer of a blood cell or the outer layer of a mesothelial cell. It is not a requirement of the patent laws to know the chemical identity of every species in the invention so long as the preferred embodiment allows one to practice the invention and obtain the utility thereof.

The Adequate Description Rejection

Claims 30-49 have been rejected under 35 U.S.C. § 112, first paragraph, for not adequately describing the transitional phrase and heparan sulfate.

With regard to the transitional phrase, the Examiner asserts that the term "consists of" is not described in the specification. Applicants respectfully disagree. While the words "consists of" may not be present in the specification, this fact does NOT mean that the transitional phrase "consists of" cannot be used. The phrase "consists of" is a legal term that has inherent support in the word "containing" (numerous instances including page 3, line 32 in the specification). In other words, changing the transitional phrase from "comprising" to "consisting essentially of" or to

"consists of" is a legal maneuver, and it is NOT necessary to have the words "consisting essentially of" or "consists of" in the specification to make such an amendment.

Furthermore, the general description of the hemocompatible surface in the specification provides support for the amended claim of a hemocompatible surface consisting of at least one of an artificial compound, a natural organic compound, or an inorganic compound and constituents of the outer layer of a blood cell, constituents of the outer layer of a mesothelial cell or a combination thereof. It should be noted that the specification (see, for example, page 6, lines 22-24) recites to "constituents" and originally filed claim 1 also recited "constituents." Therefore, the specification as originally filed provides adequate support for the claims in their present form, and indicates that the inventors had possession of the invention at the time of filing.

The First Art Rejection

Claims 30-36, 38, 39, 41-47, and 49 have been rejected under 35 U.S.C. § 102(b) or § 103(a) over Baumann et al. Baumann et al relates to endothelial cell surface heparan sulfate (ESHS) bonded to oligoamide spacers which are in turn anchored to a synthetic polymer surface. The oligoamide spacer of Baumann et al has a 16-atom chain length for the cellulose surface and an 11-atom chain length for the silicon surface.

To establish anticipation, each and every claim feature must be disclosed in a single cited art document. The independent claims require a hemocompatible surface consisting of two elements: namely, 1) at least one of an artificial compound, a natural organic compound, or an inorganic compound and 2) a constituent of the outer layer of a blood cell, a constituent of the outer layer of a mesothelial cell or a combination thereof. Generally speaking, the first element is the surface while the second element renders the surface hemocompatible.

Baumann et al fails to disclose, teach, or suggest hemocompatible surfaces with only the two required elements. In particular, Baumann et al requires the presence of oligoamide spacers. The function of the "consisting of" transitional phrase is to

EXCLUDE the use of oligoamide spacers in hemocompatible surfaces. Since Baumann et al does not disclose all of the claimed features, Baumann et al cannot anticipate any of the claims. A full copy of the Baumann et al article is enclosed to facilitate the Examiner's consideration of this rejection.

The Examiner argues that the Baumann et al oligoamide spacer bonded to a surface, e.g. silicon, is equivalent to the surface of the first element (an artificial compound) while the ESHS is equivalent to the second element which renders the surface hemocompatible. Baumann et al is a scientific paper and the limits of what are disclosed is not set forth in standard claim language. Nevertheless, reading Baumann et al to indicate that the surface co-valently modified with oligoamide spacer is equivalent to the unmodified surface of the first element of the current invention (artificial compound) is a tortured construction of what is disclosed in Baumann et al.

The process disclosed in Baumann et al explicitly requires three parts in transforming the material to hemocompatibility: a surface commonly used in the art, modification of that surface with oligoamide spacer, and only then rendering the surface hemocompatible by bonding of ESHS. Stated simply, the extra expense and effort required to make a surface co-valently modified with oligoamide spacer excludes the possibility of Baumann et al containing the same claim element as the inexpensive and readily available surface of the first element of the invention. Baumann et al fails to disclose the first element of the invention. The second element of the invention requires constituents derived from the outer layer of a blood and/or mesothelial cell. Baumann et al discloses the use of ESHS constituent derived from an ENDOTHELIAL and not a blood or mesothelial cell. Since Baumann et al does not disclose all of the claimed features, Baumann et al cannot anticipate any of the claims.

With regard to obviousness, the independent claims further stipulate the positioning of the constituents relative to at least one of an artificial compound, a natural organic compound, or an inorganic compound. Specifically, the claims require that the constituents are firmly attached to the artificial compound, the natural organic compound, or the inorganic compound by at least one of chemical immobilization,

photoimmobilization, adhesion, and drying. Baumann et al fails to teach or suggest that its ESHS is firmly attached to its synthetic polymer surface. Instead, Baumann et al teaches that its ESHS is attached to oligoamide spacers, and it is the oligoamide spacers which are attached to its synthetic polymer surface. This is important because attaching ESHS to a synthetic polymer surface, and then attaching ESH to the oligoamide spacers would NOT have suggested to one skilled in the art to firmly attach a constituent to the surface of an artificial compound, natural organic compound, or inorganic compound directly.

Furthermore, there are many disadvantages associated with using ESHS compared to using the hemocompatible constituents of the claimed invention. The preparation of ESHS requires the cultivation of endothelial cells and therefore ESHS can only be obtained in small quantities. Additionally, successful purification of ESHS is difficult to achieve. This is because the purification process is very time consuming, laborious, and cost intensive. Consequently, the combination of these factors makes ESHS coatings too expensive for commercially viable mass production. It is noted that solid phase synthesis or recombinant synthesis of ESHS is also not commercially useful as it is also too complicated and too expensive.

Furthermore the Baumann et al coating process is not commercially applicable, since ESHS can only be obtained in small quantities which are not sufficient to coat medical implants. Therefore endothelial cells have to be cultivated which is a time consuming process and also cost intensive. The endothelial cells have to be collected and the ESHS has to be isolated. The artificial or natural surface has to be modified to bear functional groups to which the linker and than the ESHS can be bound. This complex procedure may work in a laboratory but is not useful at all for commercial purposes. In this regards, the Baumann et al publication is a scientific paper of a university Professor doing basic research and explaining his complex theories, but is not a paper of a company describing the method for obtaining new hemocompatible medical devices. Until now, no company was interested in the complex technology disclosed by Baumann et al. This is because of the fact, that the Baumann et al

publication is not commercially applicable and no person skilled in the art would take a procedure which is not commercially applicable into consideration for coating medical devices. Since intellectual property rights protect new, inventive and commercially applicable inventions, the commercial applicability is one important aspect of inventions and an inventor would not use methods which are clearly not commercially applicable. Therefore a skilled artisan would refrain to take the complex Baumann et al method into consideration.

For these reasons, there are currently no products coated with ESHS available in the hemocompatible surfaces market. Therefore, ESHS is, at this time, merely an academic project and has made no significant contribution to technological progress in the field of hemocompatible surfaces.

Given the significant disadvantages of ESHS, those of skill in the art would not use ESHS as a hemocompatible coating and instead would choose to use the commercially viable synthetic heparin, since cultivation of endothelial cells and isolation of ESHS and purification of ESHS takes too much time, is too expensive, and results in too low yield which makes the ESHS not suitable for commercial use as coating material.

In addition, the independent claims state that the hemocompatible material be derived from "constituents of the outer layer of a blood cell, constituents of the outer layer of a mesothelial cell or a combination thereof." Baumann et al only discloses material obtained from aortic endothelial cells and not from blood or mesothelial cells. Therefore, Baumann et al would NOT have suggested to one skilled in the art to look towards more easily obtained blood cells and mesothelial cells as a source for hemocompatible material.

Further Baumann et al teaches the use of a pure fraction of endothelial cell surface heparan sulfate (ESHS). As explained above the claimed invention is directed to the use of multiple constituents of the outer layer of a blood cell or the outer layer of a mesothelial cell to form a hemocompatible surface. Thus the use of only the single ESHS fraction as disclosed in Baumann et al does not anticipate the claimed invention.

Nowhere does Baumann et al even suggest the use of multiple constituents of the outer cell layer. In fact Baumann et al expressly **teaches against** the use of multiple constituents. Baumann clearly states that the unfractionated heparin (HE) showed high platelet adhesion and thrombus formation *in vitro* whereas the purified ESHS fraction showed no platelet adhesion. Therefore, one skilled in the art would have been taught from Baumann et al to avoid the use of multiple constituents to form a hemocompatible coating as claimed in the invention.

Thus the use of only the single ESHS fraction as disclosed in Baumann et al does not make obvious the claimed invention of a hemocompatible coating consisting of multiple constituents of a blood cell or mesothelial outer layer.

In contrast to Baumann et al, the claimed invention provides a significant step forward in technological progress in the field of hemocompatible surfaces. The claimed invention provides a fully hemocompatible surface that is not only simple to produce, but is also produced from source materials that are cheap and available in large quantities.

In light of the differences between the claims and Baumann et al, one skilled in the art would not have been motivated by Baumann et al to make the novel hemocompatible surfaces of the claims. And since Baumann et al does not teach or suggest hemocompatible surfaces consisting of two elements: namely, 1) at least one of an artificial compound, a natural organic compound, or an inorganic compound not hereto subjected to any special chemical treatment and 2) constituents of the outer layer of a blood cell, constituents of the outer layer of a mesothelial cell or a combination thereof, and since Baumann et al does not teach or suggest the relative positioning of these two elements, Baumann et al cannot render the claims obvious.

Moreover, unexpected advantageous effects of the claimed glycocalyx constituents (constituents of the outer layer of mesothelial cells or blood cells) over the ESHS of Baumann et al can be shown. A test for thrombogenicity was used wherein an artificial surface was coated either with ESHS or with our glycocalyx constituents. The ESHS coated surface showed a reduction of platelet aggregation on the surface of

about 50% in comparison with an uncoated surface. Surprisingly, the surface coated with the inventive constituents of the outer layer of mesothelial cells or blood cells showed a reduction in platelet aggregation of about 80% in comparison to the uncoated surface. The platelet aggregation is linear correlated with the thrombogenicity.

A possible explanation for this unexpected result could be that platelet aggregation is initiated by different proteins in the blood which adsorb to the non-hemocompatible surface. These proteins comprise, for instance, albumin, immunoglobulines, fibronectin, fibronogen, high molecular weight fibrinogen. In the case that the non-hemocompatible surface is coated with ESHS, the aggregation of some but not all of the proteins is prevented, which initiate platelet aggregation. However, if the non-hemocompatible surface is coated with all constituents of the glycocalyx, the adhesion of all proteins can be prevented and consequently the platelet aggregation is lower in comparison to the surface coated with ESHS.

The Second Art Rejection

Claims 37, 40, and 48 have been rejected under 35 U.S.C. § 103(a) over Thompson in view of Baumann et al. Thompson relates to making a prosthesis that is made of metal, polymeric monofilaments or polymeric multifilament yarns. Thompson fails to teach or suggest hemocompatible surfaces consisting of two elements: namely, 1) at least one of an artificial compound, a natural organic compound, or an inorganic compound and 2) constituents of the outer layer of a blood cell, constituents of the outer layer of a mesothelial cell or a combination thereof. Thompson also fails to teach or suggest the relative positioning of these two elements.

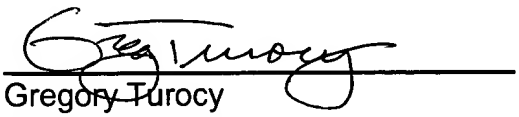
Claims 37, 40, and 48 are patentable because Thompson does not cure the deficiencies of Baumann et al with regard to the independent claims. Consequently, claims 37, 40, and 48 are patentable for the same reasons that claims 30, 39, and 47 are patentable.

Should the Examiner believe that a telephone interview would be helpful to expedite favorable prosecution, the Examiner is invited to contact Applicants' undersigned attorney at the telephone number listed below.

In the event any fees are due in connection with the filing of this document, the Commissioner is authorized to charge those fees to our Deposit Account No. 50-1063.

Respectfully submitted,

**AMIN & TUROCY, LLP**



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# Which Glycosaminoglycans Are Suitable for Antithrombogenic or Athrombogenic Coatings of Biomaterials? Part II: Covalently Immobilized Endothelial Cell Surface Heparan Sulfate (ESHS) and Heparin (HE) on Synthetic Polymers and Results of Animal Experiments

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*ABSTRACT* **A systematic study was performed on immobilizing unfractionated heparin and endothelial cell surface heparan sulfate covalently with the spacer concept onto two polymer surfaces, followed by characterization of the surface concentration and in vitro and in vivo platelet adhesion properties under comparable high shear rates for microvascular vessels.**

**Oligoamide spacer with a 16-atom chain length on cellulose surface and an 11-atom chain length on silicon surface, respectively, was used for immobilizing HE and ESHS via amino groups of glucosamine to the spacer which was anchored to the polymer surface. The surface concentration was in the range of 7 to 10 pmol/cm² for HE and 1 to 1.5 pmol/cm² for ESHS. This is in agreement with a calculated monolayer covering of ESHS and HE. In vitro and preliminary in vivo measurements (beagle, sheep) showed no platelet adhesion on the ESHS coatings, whereas HE showed high platelet adhesion and thrombus formation in vitro as well as in vivo. ESHS coating may be a potential candidate for preparing smooth artificial microvascular blood vessels.**

*Keywords:* **Heparin, endothelial cell surface heparan sulfate, platelet adhesion, surfaces, animal experiments**

The role of different types of glycosaminoglycans (GAGs) involved in blood coagulation and their potential as electrostatically bound coating material on polymer surfaces to prepare biocompatible and hemocompatible biomaterials has been reported in Part I.[1] It was shown that electrostatically bound GAGs on partially cationized cellulose membrane surfaces can be used as releasing system for short-term implants or biomaterials, as long as the GAG reservoir enables achieving the desired GAG concentration until complete desorption from the surface occurs.

At the chosen high shear rates (1050 $sec^{-1}$), similar to those in microvascular vessels, a high rate of platelet adhesion and reaction was prominent in vitro for heparinized surfaces as well as for surfaces coated with hyaluronan, chondroitin sulfate, keratan sulfate, or dermatan sulfate. ESHS-coated surfaces did not show any platelet adhesion. In this article we report on the potential of type 2 GAG HE and ESHS as covalently immobilized coating material for preparing long-term implants, such as artificial vessels.

The spacer concept for HE covalently linked to polymer surfaces was used in such a way that HE retained its ability to form a complex with plasma antithrombin and thus behave like heparin in solution.[2-10] Comparable immobilization conditions were employed

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for ESHS, using the spacer concept. The amount of immobilized GAGs on the polymer surface was analyzed via glucosamine content and in vitro platelet adhesion test, at defined shear rates of 1050 $sec^{-1}$. Some results of animal experiments are reported for developing artificial microvascular blood vessels.

## MATERIALS AND METHODS

### Materials

Chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Munich, Germany) in p.a. quality. Cellulose dialysis tubes were from Serva (Heidelberg, Germany); Cuprophan® was a gift from Enka Akzo (Glanzstoff). 3-Chloro-2-hydroxypropyl-trimethylammonium chloride (CHMAC) was obtained from Degussa (Frankfurt, Germany), and, HE grate I was from Serva. Silicone tube was a gift from Fresenius AG (St. Wendel, Germany) and vessel connectors were a gift from Professor Losert (Wien, Austria); glass connectors were prepared from 6-cm-long glass tubes with a luminal diameter of 3 mm.

### Endothelial Cell Culture

Endothelial cells were prepared from bovine aortas and cultured in DMEM in untreated culture flasks as described previously.[1,11]

### Isolation of ESHS

ESHS was isolated from medium of endothelial cell cultures according to several chromatographic methods as described earlier.[1,11,12]

### Characterization of ESHS

The isolated ESHS was characterized by HPLC/GPC, glucosamine content,[13–15] sulfation degree, disaccharide content, and domain structure. The criteria for purity were comparable to those reported earlier.[1,12]

### Partially Cationized Cellulose Membranes

Partially cationized cellulose membranes (CHMAC-cellulose) were prepared as described earlier.[13]

### Synthesis of Substituted Carboxylmethyl Cellulose Containing Oligoamide Spacer Groups

Partially substituted carboxymethyl cellulose membranes (CMC) were prepared by modifying cellulose with monochloroacetic acid in ethanol for 8 hours, followed by washing with 10% HCl. To create the carboxyl-terminated spacer, the CMC was reacted first with hexamethylenediamine and then with adipic acid in a solution of 0.1% *N*-cyclohexyl-*N'*-2-(morpholinoethyl)-carbodiimide-methyl-*p*-toluene sulfonate (CME-CDI) in 2-(*n*-morpholino)ethane sulfuric acid buffer (MES), pH 4.75, over 30 minutes at 4°C. The molar amount of the carboxyl groups of the spacer was estimated by a conductometric method.[16]

### Immobilization of GAGs on Carboxyl Group-Terminated Cellulose Spacer Groups

Reaction of GAGs with the carboxyl groups of the spacer was carried out in a solution of 0.1% CME-CDI in MES, pH 4.75, over 30 minutes at 4°C. The GAG modified cellulose membranes were rinsed with ice water and hydrolyzed with 8 N HCl over 4 hours for chromatographic analysis.[16]

### In Vitro Blood Tests

In vitro blood tests at laminar flow and shear rates of 1050 $sec^{-1}$ were performed in a Baumgartner perfusion chamber modified by Sakariassen et al,[17] as reported earlier.[1,13,15]

### Animal Experiments

A 16-month-old male beagle was used in the first part of the animal experiments. Enflurane was used for anesthesia throughout the entire experiment. The membranes were sterilized with 25 kgray radiation and then fixed on a fiber by knots. The membranes fixed on the fiber were placed in the left auricle for 9 hours.[18]

Austrian sheep (1 1/2 to 2 1/2 years old) were anesthetized with 1 ng atropin, 10,000 IU HE was used only for surgery purposes. One-meter-long steam-sterilized silicone tubes (uncoated, HE or ESHS coated),[12] 3 mm Ø, were fixed via a vessel connector or via an ESHS-coated glass connector to the carotid artery and vena jugularis. The blood flow was measured with ultrasound. The experiment was stopped when reduced blood flow was encountered. AV shunts were explanted with citrated buffer, fixed with glutaraldehyde, and stained with 90% methanol for 5 minutes, 50% May-Grünwald solution for 5 minutes, and 20% Giemsa solution for 20 minutes. The relative platelet coverage was estimated microscopically with a light microscope (Olympus CK2) and a grid of lines (Leitz) on the inner surface of the dissected tube and along the dissected tube. Further details will be published separately.

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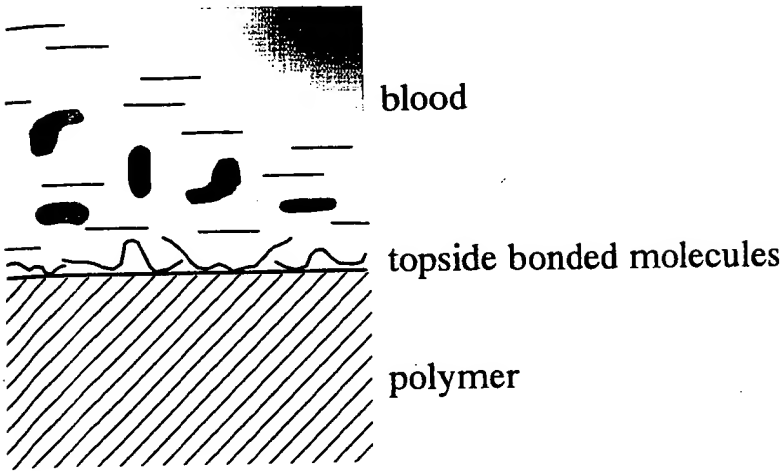


FIG. 1. Rendering of the interaction of biomaterials with smooth surfaces and top side covalently bonded molecules with blood.

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## RESULTS AND DISCUSSION

Molecules on the top side of medical devices, implants, or biomaterials are responsible for their biological properties influenced by the interaction of the surface molecules with body fluids such as blood (so-called biocompatibility or hemocompatibility). In contrast, molecules in the interior of medical devices are responsible for the mechanical properties, so-called biomechanical properties (Fig. 1).

Whereas the mechanical properties of implants have mostly been solved (PU heart valve bladders are stable several years 40 Mio strokes/year),[19] the long-term surface properties are not satisfactory with regard to a high hemocompatibility standard as in native blood vessels.[15,20] Surface-modified implants with short-term improved hemocompatible properties have been developed by using a releasing system (eg, electrostatically bound polymer).[1,20] Long-term properties may be achieved with different surface modification concepts, especially via covalent bonds, because depot concentrations cannot be made infinitely high.

There have been attempts to improve the properties of artificial smooth surfaces of long-term medical devices by introducing covalently bonded hydrophilic, hydrophobic, charged, and hydrogel structures as well as anticoagulants, fibrinolytics, enzymes, and plasma proteins to improve the hemocompatibility standard.[14,20–22] However, the high hemocompatibility standard of surfaces of natural blood vessels has not yet been reached.[20]

Most work has used HE,[23–34] an anticoagulant that belongs to the type 2 GAGs.[1] However, we could show that ESHS, also with a heparan backbone, but different fine structure, belongs to the type 2 GAGs. It seems to have potential for preparing artificial surfaces of hemocompatibility standard comparable to natural blood vessels,[11–16,18,22,35] as can be seen in Figure 2.

ESHS is the main component of the plasma membrane of endothelial cells; the latter covers the inner luminal surface of blood vessels as the monocellular lining (Fig. 2, left side below).

The ESHS polysaccharide chains (36 kDa) are anchored via a 56-kDa protein core to the plasma membrane, probably via a phosphatidylinositol bond. ESHS probably belongs to the glypican type. This endothelial cell surface proteoheparan sulfate (ESPHS) can be released from cultured endothelial cells or fermented endothelial cells by shedding. The protein core can be removed by β-elimination or proteolytic degradation.[1] The isolated ESHS can be immobilized via cross-linker or the spacer concept to artificial surfaces, such as artificial vascular grafts (Fig. 2, right side below). Another pathway for isolation of ESHS (Fig. 2, middle) is a lung perfusion system, using proteolytic enzymes for releasing ESHS during perfusion. When the protein core of ESPHS is removed by enzymatic digestion or β-elimination, ESHS from different sources may be used. There was no difference in molecular weight between lung ESHS, bovine aorta ESHS,[12] and human umbilical vein endothelial $H_5$36. It seems that ESHS does not form monoclonal antibodies.

In this study we immobilized HE type 2 GAG according to Figure 3 with the help of flexible spacer molecules covalently bound to the polymer surface in such a way that it retained its ability to form a complex with plasma antithrombin and thus to behave like HE in solution. This effect may also be similar to end group-bonded heparin.[2–10]

Amide bonds which are hydrolytically relatively stable were formed via free amino groups of HE and carboxyl group-terminated oligoamide spacer, for covalent binding of HE or ESHS to cellulose via oligoamide spacer, again with hydrolytically relatively stable amide bonds. Cellulose membrane was partially reacted with monochloroacetic acid, resulting in partially substituted carboxymethyl cellulose (CMC).[16] The second step was the reaction of one amino group of hexamethylenediamine with the carboxyl groups of partially substituted

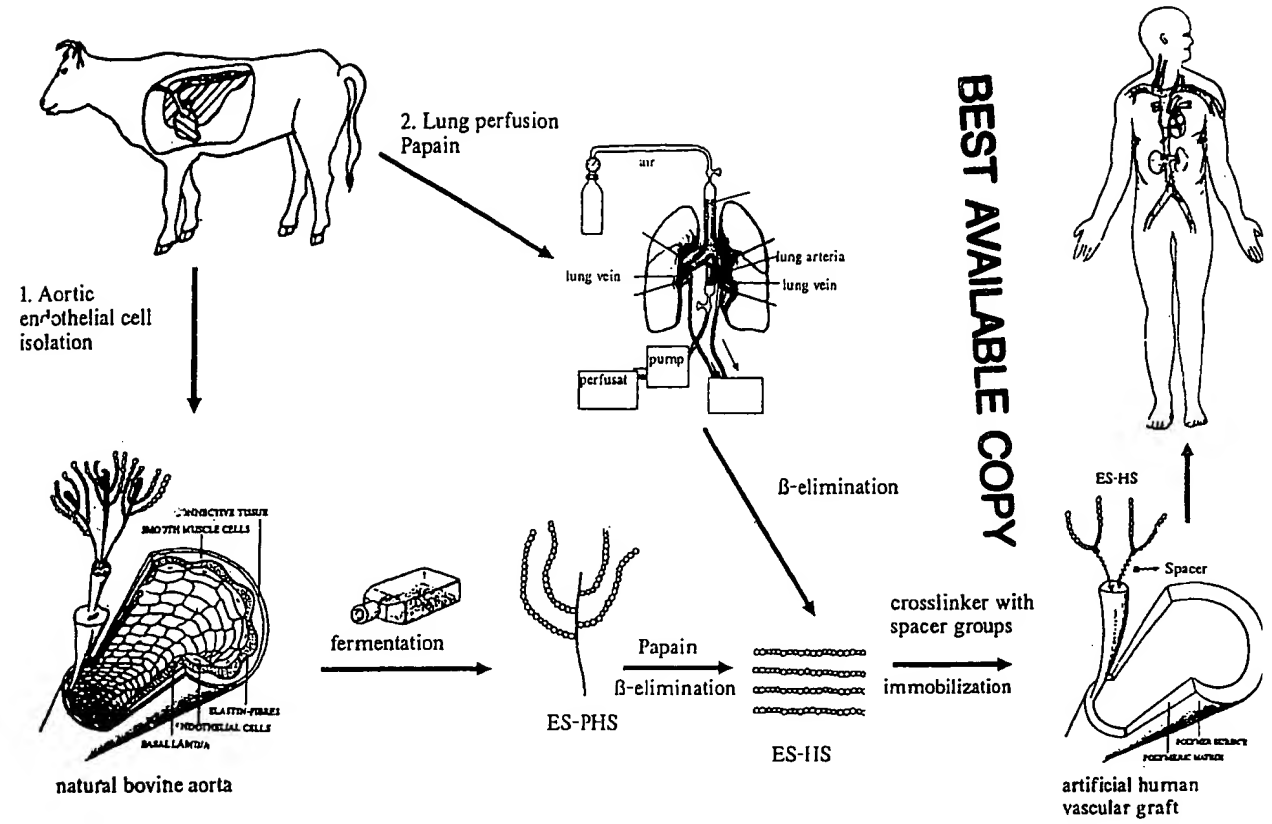


FIG. 2. Baumann/Keller concept for the preparation of hemocompatible implants and medical devices by surface modification wit ESHS.

CMC membrane, using CME-CDI forming an amide bond.[16] The third step was the reaction of the terminated amino groups with the carboxyl group of adipic acid with the help of CME-CDI. The fourth step was the formation of amide bonds between the carboxyl and the amino groups of HE or ESHS. The spacer length was 1.85 nm in expanded version and had 16 atoms as spacer constituents[16] (Fig. 3).

Comparable immobilization conditions were used for the immobilization of ESHS employing the concept shown in Figure 3 so as to compare the results of bloo tests. The amounts of immobilized ESHS and hepari on the cellulose membranes with oligoamide spacers ar shown in Table 1.

The concentration of ESHS and HE on the polyme surface was determined via anion exchange chromatogra phy with pulsed amperometric detection by analysis of th glucosamine peak from hydrolyzed modified cellulose.

The amounts of immobilized ESHS were in th same range and for HE slightly higher than theoreticall

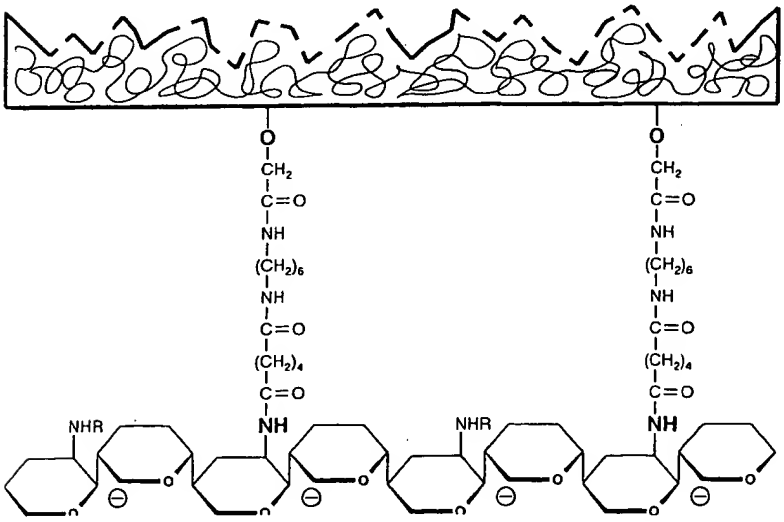


FIG. 3. Scheme for covalent immobilizatior of HE and ESHS using the oligoamide spacer concept.

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**TABLE 1. Results of Concentration of Immobilized HE and ESHS on Cellulose Surface with Oligoamide Spacer Length of 1.85 nm in Expanded Version, Compared with Theoretical Side-to-Side Coordination for a Monolayer**

| Spacer | Spacer Length | | COOH Groups | Heparin (13 kDa) | ESHS (36 kDa) |
|---|---|---|---|---|---|
| | nm | Atoms in Chain | (nmol/cm²) | (pmol/cm²) | (pmol/cm²) |
| Carboxymethyl-hexamethylenediamine-adipic acid | 1.85 | 16 | 11.5 | 10.3 | 1.1 |
| Theoretical | | Side to side | | 3.6–7.7 | 1.1–2.4 |
| Calculated | | End on | | 157.0 | 157.0 |

calculated for the formation of a monolayer on surfaces with side-to-side coordination.[1,37–39]

## In Vitro Platelet Adhesion Test

The percent of platelet adhesion was measured on cellulose membranes which were fixed on a cover slip and placed in the Baumgartner perfusion chamber modified by Sakariassen et al.[17] Whole citrated blood was perfused for 5 minutes at shear rates of 1050 $sec^{-1}$, comparable to microvascular vessels.

The results of these tests are seen in Figure 4. CHMAC-cellulose showed 100% platelet adhesion, unmodified cellulose 5%, oligoamide spacer-coated cellulose (chain length 1.85 nm and 16 atoms) 30%, heparin-anchored oligoamide spacer 50 to 60%, and ESHS-anchored oligoamide spacer 0%.

Again, ESHS covalently bound to a polyamide spacer showed no platelet adhesion, whereas platelet adhesion was prominent (50 to 60%) on covalently bonded heparin.

## Animal Experiments

First screening studies for testing the covalent HE and ESHS coating were performed in the left auricle of an anesthetized male beagle.[18,22] This experiment was not a standard animal experiment. The beagle was used at the same time for other surgery studies which did not influence the results of adhesion of plasmatic and cellular components to modified cellulose membrane surfaces.

Five different modified cellulose membranes were used[18,22]:

- Unmodified cellulose membrane
- Partially cationized cellulose membrane
- Cellulose containing oligoamide spacer groups (16 atoms, length 1.85 nm)

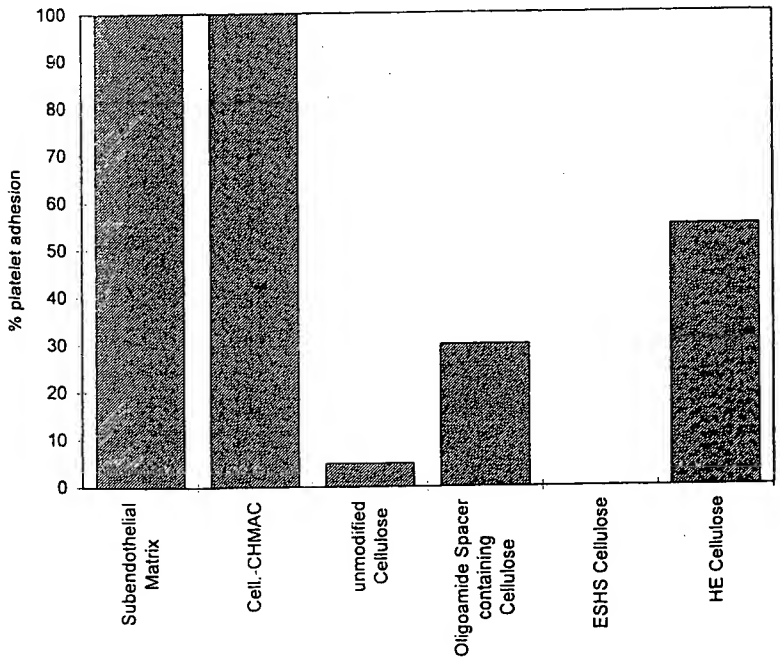


**FIG. 4.** Percent platelet coverage on unmodified, spacer-containing CHMAC, HE, and ESHS modified cellulose membranes.

- Heparin + oligoamide spacer + cellulose
- ESHS + oligoamide spacer + cellulose

It cannot be ruled out that during the animal experiment, plugs of adherent red and white thrombi may have been released by the high turbulence from the membrane surface which can be partially seen on heparinized membranes.

The shear rate and turbulence inside the left auricle were not measured. They should be extremely high, higher than the laminar flow shear rates in microvascular vessels, which were selected for the dynamic in vitro blood tests in the flow chamber. The results of animal experiments are presented in Figure 5.

The high thrombogenicity of heparinized, CHMAC spacer groups containing an unmodified cellulose membrane without using anticoagulants is not surprising. It is known that with increasing shear rates, higher than 600 $sec^{-1}$, platelet adhesion reactivity, aggregation, and morphologic changes become more prominent.[40] The difference in the results may be explained by the different reactivity of the top layer molecules on the membrane surface itself. This was also found in tests in the perfusion chamber with citrated blood.

We have to point out that in the literature, although not very often, an agreement between results of in vivo and in vitro measurements has been reported.[20,41] We could not find major differences among results of bloo tests of ESHS coatings under in vitro and in vivo condi tions for covalently bound ESHS via a spacer which i optimal for HE immobilization concerning the lengt and flexibility of the spacer and the conformation of HE There were also no differences between ionically boun ESHS as long as there was enough ESHS reservoir o the polymer surface, as previously reported.[1,13,22] Thes preliminary results indicate that an ESHS coating ma be suitable for preparing athrombogenic polymers with similar high standard of hemocompatibility as in nature In contrast, covalent heparin coatings, under our experi mental conditions, were not suitable at high shear rate for preparing hemocompatible long-term biomaterials.

Partially cationized cellulose membranes are extremely thrombogenic both in vitro[1,13,22] and in vivo. The higher standard of thrombogenicity of unmodified cellulose membranes under in vivo conditions has not been found under in vitro conditions. This additional effect in vivo may derive from the higher potential of aggregation and reactivity of platelets at the probably extremely high turbulence in the left auricle. Similar arguments are valid for cellulose membranes with spacer groups.

The results of platelet adhesion and reactivity to heparinized surfaces are difficult to explain. However, they seem to be in agreement with literature results.

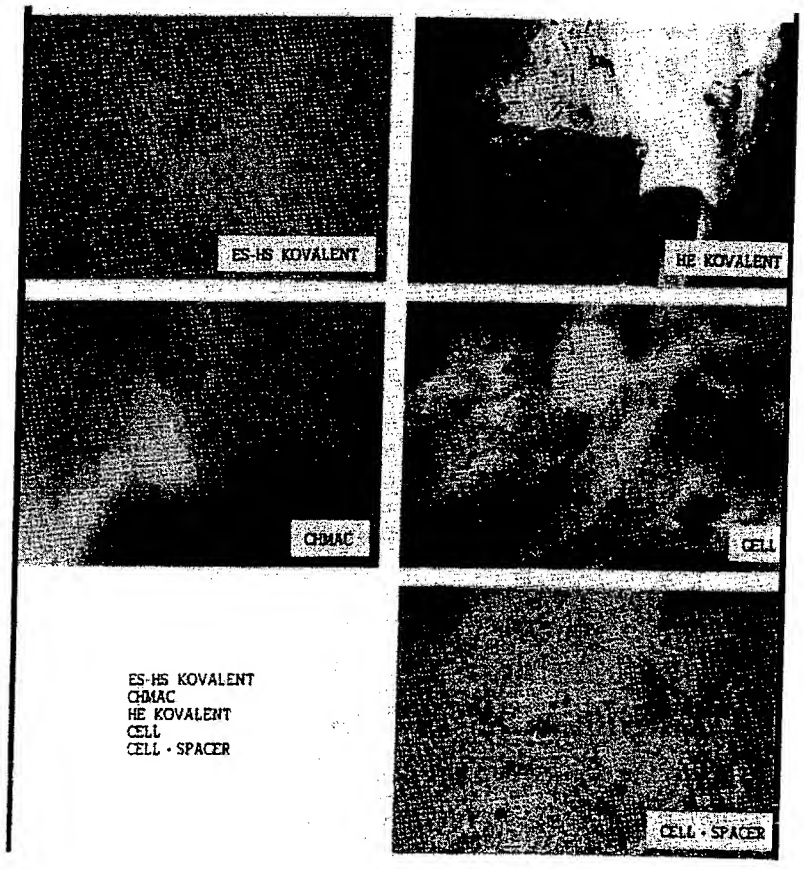


FIG. 5. Animal experiments in the left auricle of a male beagle of unmodified, partially cationized cellulose containing oligoamide spacer groups and HE- and ESHS-coated cellulose membranes.

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The extreme shear conditions (1050 $sec^{-1}$) for in vitro tests are comparable to those where white fibrin clots, platelet accumulation, and platelet reaction are prominent. At low shear rates or sluggish blood flow, fibrinogen adsorption or red fibrin clot formation is more prominent, according to Salzman et al.[20] It is known that heparinized surfaces adsorb platelets and induce platelet aggregation and secretion.[42,43] These effects may be inhibited partially by antithrombin because heparin–antithrombin complexes have been described to be more passive toward platelets.[20,44] It must be pointed out that the commercial heparin used is heterodisperse (13 kDa) and the low-molecular-mass subfraction of heparin (5 kDa) was not used in our experiment. This has been described as having the least potential for undesirable interactions with platelets.[42] However, heparin fractions with high AT III affinity do adsorb fibrinogen preferentially[45] and adsorbed fibrinogen again is predominately responsible for platelet adhesion.[46] Fibrinogen is a cofactor for platelet aggregation in which the protein is thought to bind to glycoprotein receptors on the platelet surface to form a bridge between continuous platelets.[47] Additionally it has to be taken into account that heparin binds to many other plasma proteins[48] and components of the extracellular matrix, whereas ESHS does not bind irreversibly to these components or fibrinogen under strong physiological conditions.[35,45] Thus, surfaces coated covalently with heparin at high shear rates, are a good tool for the rapid adhesion of different plasma proteins and platelets. This effect induces a growing new surface where the heparin coating is completely covered with natural proteins and cellular components. The diameter of the artificial microvessels will consequently be reduced if no desorption takes place. ESHS properties seem to be such that no irreversible binding of proteins and platelets apparently occurs under physiological conditions.

The results are preliminary and have to be repeated under the scrutiny of statistics and properly planned animal experiments. However, the results are promising for developing microvascular vessels using, for example, smooth silicone tubes and the covalent ESHS coating principle.

## Development of Microvascular Vessels

Vascular disease, such as atherosclerosis, is usually progressive. No organization is marketing arterial grafts suitable for the replacement of coronary arteries. Presently, surgeons still harvest saphenous vein grafts from a patient and are interested in a viable off-the-shelf arterial substitute. Thus, a major new market opportunity exists in the development of an improved small-diameter (3 mm or less) vascular prosthesis.[19] Two biomaterials dominate the vascular graft market: Dacron (woven) and PTFE (blown) with topologic differences on the surface which cause the formation of pseudoneointima and reduce the diameter.[49] Only biomaterials with smooth surfaces are suitable for development of artificial blood vessels with a comparable high hemocompatibility standard as in nature. It seems to be very important that these surfaces not irreversibly adsorb major plasma proteins as described above. Otherwise, blood flow would be reduced or stopped.

For this development we selected a 3-mm-diameter silicone tube with a smooth surface and anchored HE and ESHS, respectively, via an oligoamide spacer (12 chain atoms, 1.4 nm) onto the inner luminal side of the silicone tube. The amounts of covalently immobilized ESHS (1.5 $pmol/cm^2$) and HE (7.2 $pmol/cm^2$), respectively, were similar to side-to-side coordination as can be seen in Table 2.[37–39]

This means that there is enough HE or ESHS on the inner luminal side of the silicone tube to form a monolayer without having uncoated parts on the silicone surface. Unpublished results from in vitro pump tests at high shear rates with whole blood of healthy volunteers showed no platelet adhesion to ESHS-coated silicone tubes and a relatively high platelet adhesion to heparin-modified surfaces. The latter is consistent with a high platelet loss.

Animal experiments on a female Austrian sheep with artificial blood vessels implanted in the form of an AV shunt (carotid artery and vena jugularis), 1-m-long ESHS-coated silicone tube connected via vessel connectors to the carotid artery and the vena jugularis, showed no platelet coverage after 2 and 18 hours test time, whereas heparinized silicone tubes showed a 50%

**TABLE 2. Results of Concentration of Immobilized HE and ESHS on Silicone Surfaces with Oligoamide Spacer Length of 1.4 nm in Expanded Version and 12 Atoms as Spacer Constituents, Compared with Theoretical Side-to-Side Coordination for Monolayer**

| | *Spacer Length* | | | | |
|---|---|---|---|---|---|
| *Spacer* | *nm* | *Atoms in Chain* | *COOH Groups ($nmol/cm^2$)* | *Heparin (13 kDa) ($pmol/cm^2$)* | *ESHS (36 kDa) ($pmol/cm^2$)* |
| Aminopropylsiloxy-adipic acid | 1.4 | 11 | 8.85 | 7.5 | 1.5 |
| Theoretical | | | Side to side | 3.6–7.7 | 1.1–2.4 |
| Calculated | | | End on | 157.0 | 157.0 |

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relative platelet coverage (white thrombi) and a reduced blood flow after 2 hours. After 18 hours the blood flow was stopped. Unmodified silicone tubes showed a 20% platelet coverage after 2 hours and 40% after 4 hours.[12] The results of long-term AV sheep experiments with ESHS-coated glass connectors and ESHS-coated silicone tubes (1 m long, 3 mm Ø) and without using anticoagulants, except at the beginning of the experiment, showed no reduced blood flow after 400 days and no platelet adhesion. In contrast, blood flow in the AV shunt of uncoated silicone tubes stopped after 12 days, and the silicone tube was completely covered with thrombi, whereas ESHS-coated glass connectors were completely free of platelets.[12]

Again, ESHS is the likely favorite for developing long-term athrombogenic microvascular vessels, whereas surfaces with covalently immobilized heparin show high platelet adhesion and reactivity at high shear rates in short-term animal experiments. The results are consistent with arguments presented earlier and results from the literature. In contrast, in clinical use, surfaces heparinized by electrostatic binding have been successfully employed in short-term applications (eg, subclavian-to-femoral artery shunt) but have performed erratically in long-term applications (eg, heart valves.[1,21]) The used Heparin is suitable as a releasing system at low shear rates,[1,21] whereas ESHS coating is tailored in nature for high shear rates. The isolation procedure and covalent immobilization of ESHS on polymers seems to be mild enough so as not to change the favorable biological and clinical properties of ESHS, when ESHS is applied as a coating for artificial blood vessels.

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